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An in vitro model system that can differentiate the stages of DNA replication affected by anticancer agents

Waleed Abdel-Aziz*, Robert J. Hickey, Linda H. Malkas

Department of Medicine, Hematology/Oncology Division, Indiana University School of Medicine, Indiana University Cancer Research Institute, 1044 W. Walnut Street, R4-202 Indianapolis, IN 46202, USA

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Abstract

We have previously reported on the potential use of a novel in vitro human cell-derived model system to investigate the mechanism of action of anticancer agents that directly affect the process of DNA replication. Our cell-free system uses a multiprotein DNA replication complex (designated the DNA synthesome) that has been isolated, characterized, and extensively purified from a wide variety of mammalian cells and tissues. The DNA synthesome is competent to orchestrate simian virus 40 (SV40) origin-specific and large T antigen-dependent DNA replication in vitro. In this study, the synthesome-based cell-free system was tested to evaluate the mechanism of action of 1-β-D-arabinofuranosylcytosine (ara-C), camptothecin (CPT), and doxorubicin (DOX). Using a novel synthesome-based in vitro kinetic assay, we demonstrated that DNA replication mediated by the synthesome is initiated within the SV40 replication origin and proceeds bidirectionally in a manner analogous to that occurring within the cell. Ara-CTP, CPT, and DOX have been found to affect different stages of the in vitro DNA replication process mediated by the complex. Ara-CTP inhibited both the initiation and elongation stages, whereas CPT produced most of its effects by inhibiting the elongation phase of DNA replication. DOX inhibited the termination stage of DNA synthesis mediated by the synthesome. The data presented here support our contention that the DNA synthesome represents a highly effective in vitro model system for investigating the mechanism by which some anticancer agents can directly affect the process of DNA replication.

Keywords: DNA synthesome; DNA replication; Kinetic assay; SV40; Anticancer agents; In vitro model system

1. Introduction

Due to the rapid progress in the field of cancer chemotherapy and the continued synthesis of agents that arrest the growth of cancer cells through the inhibition of the DNA replication process, there is a compelling need for an in vitro system that models the cellular DNA synthetic machinery and can be used for rapid and accurate analysis of the molecular actions of these compounds. In this regard, we have previously utilized an in vitro model system to investigate the mechanism of action of S-phase specific anticancer agents [1–6]. Our cell-free system uses a multiprotein DNA replication complex (designated the DNA synthesome) that has been isolated, characterized, and extensively purified from a wide variety of mammalian cells as well as from tissues. The replication proteins identified to copurify with the DNA synthesome include DNA polymerases α, δ, and ϵ, DNA primase, replication protein A (RPA), replication factor C (RFC), proliferating cell nuclear antigen (PCNA), DNA ligase I, DNA helicase, poly(ADP-ribose) polymerase (PARP), DNA methyltransferase (DMT), and topoisomerases I and II [7–14]. We have shown that the DNA synthesome is a discrete protein species in 4% native polyacrylamide gels [13,15]. In addition, the integrity of the DNA synthesome is maintained after its treatment with salt, detergent, RNase, DNase, anion exchange chromatography, sedimentation through glycerol or sucrose gradients and electrophoresis through non-denaturing polyacrylamide gels; indicating that the association

Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CTP, 1-β-D-arabinofuranosylcytosine triphosphate; CPT, camptothecin; DOX, Doxorubicin; SV40, simian virus 40; ori, origin of DNA replication; T Ag, SV40 large T antigen

* Corresponding author. Tel.: +1-317-278-4229; fax: +1-317-274-0396.
E-mail address: wabdelaz@iupui.edu (W. Abdel-Aziz).

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of the replication proteins with one another is independent of non-specific interaction with other cellular macromolecular components. Most importantly, the DNA synthesome is fully competent to support SV40 origin-specific DNA replication in vitro in the presence of large T antigen [3,7,9,12,13]. This in vitro ability of the synthesome is effectively equivalent to that observed for cells permissive for SV40 infection [16]. SV40 is totally dependent on the cell's DNA replication machinery for the duplication of its DNA genome. This fact, coupled with our other data, strongly suggests that the DNA synthesome is the minimal cellular machinery required to carry out mammalian cell DNA replication.

In a previous study [3], we demonstrated that ara-CTP inhibited the formation of DNA replication intermediates during the in vitro replication reactions mediated by the DNA synthesome. This action was accompanied by a preferential inhibition of DNA polymerase α, and not DNA polymerase δ, at the mammalian DNA replication fork [3,5]. The reaction products formed by the DNA synthesome in the absence or presence of ara-CTP were essentially identical to those formed by intact cells [2,3]. We have also demonstrated that the DNA synthesome could utilize ara-CTP as a substrate and incorporate it into internucleotide linkages [2,6]. A correlation was found to exist between the IC_{50} values of ara-C and CPT required to inhibit cancer cell DNA synthesis and that required for inhibition of synthesome-mediated in vitro DNA replication [1,3]. In addition, Coll et al. [1] found that CPT inhibited the activity of topoisomerase I associated with the synthesome with a concentration-dependent increase of synthesome-associated topoisomerase I–DNA cleavage complexes. The increase in cleavage complex formation occurred in a manner similar to that produced by purified enzymes.

In the following described study, we extend our findings to more clearly and finely define the inhibitory mechanism of ara-C, CPT, and DOX using the synthesome-based in vitro model system. These clinically relevant anticancer agents were selected to serve as representative chemotherapeutics to evaluate our cell-free system, and were considered good candidates for our studies because they are S-phase specific agents, have an extensive literature base describing their mechanisms of action, and target three different enzymes (DNA polymerase α, topoisomerases I and II, respectively) which are among the components of the DNA synthesome. We also report the development of a novel kinetic assay to determine precisely which stage(s) of the DNA replication process, mediated by the DNA synthesome in vitro, is (are) affected by these drugs. The results support our contention that the human cell DNA synthesome is a valid model system for investigating the mechanism(s) by which anticancer drugs that selectively target the DNA replication process in vitro, also, exert their effects on this process in intact cells.

2. Materials and methods

2.1. Materials

Ara-CTP, DOX, vincristine (VCR), and non-radioactive nucleotides were purchased from Sigma Chemical Company. CPT was obtained from TopoGEN, Inc. Restriction endonucleases were purchased from New England Biolabs. The plasmids pUC.HSO (ori^+) and pUC.8-4 (ori^-) were kindly supplied by Dr. Richard Piehl. [α-^{32}P]dATP and [α-^{32}P]dGTP were obtained from Perkin-Elmer Life Sciences, NEN.

Ara-CTP, DOX, and VCR were dissolved in 10 mM HEPES (pH 7.5) while CPT was dissolved in DMSO. Aliquots of each drug were stored at −80 °C as 10 mM stock solutions and diluted before each experiment.

2.2. Cell culture and harvest

Suspension cultures of the breast cancer cell line MDA MB-468 were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated calf and fetal bovine serum. Monolayer cultures of MCF-7 cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine. Exponentially growing cells were harvested and washed three times with PBS comprising 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.8 mM Na_2HPO_4. The cells were then pelleted by low-speed centrifugation and the cell pellets were stored at −80 °C until subjected to subcellular fractionation.

2.3. Isolation and purification of the DNA synthesome from breast cancer cells

The DNA synthesome was isolated from breast cancer cells (MDA MB-468 and MCF-7) according to our published procedures [7,9].

2.4. Purification of SV40 large T antigen

SV40 large T antigen was purified according to the method of Simanis and Lane [17].

2.5. In vitro SV40 DNA replication assay

The reaction was carried out according to the procedures of Malkas et al. [7] and Abdel-Aziz et al. [3]. To determine the amount of radiolabel incorporated into the daughter DNA molecules produced during the reaction, the reaction mixture was spotted on Whatman DE81 filters and quantified by liquid scintillation counting [18]. For gel analysis of the replication products, 1% agarose gels containing either TBE (90 mM Tris/90 mM boric acid/2 mM EDTA) or alkaline (50 mM NaOH/1 mM EDTA) buffer were used. Alkaline gels were soaked in 7% trichloroacetic acid for

45 min at room temperature before the gels were dried and exposed to Kodak XAR-5 films at −80 °C to visualize the reaction products.

2.6. Restriction endonuclease digestion of pSVO$^+$ plasmid

Studies were initiated to search for a combination of restriction enzymes each with a unique restriction site in the pSVO$^+$ plasmid that was capable of completely digesting the DNA in the presence of other endonucleases. The selected restriction enzymes produced five DNA fragments ranging in size from 411 to 900 bp. pSVO$^+$ plasmid (500 ng) was incubated with the restriction endonucleases *Psh*A I, *Ava* I, *Alw*N I, *Bsa* I, and *Sca* I for 1 h at 37 °C followed by another hour at 50 °C. The DNA digestion products were resolved by 1.5% agarose gel electrophoresis in TBE buffer. The gel was stained with ethidium bromide (0.5 μg/ml) and the DNA fragments were visualized by illuminating the gel with an ultraviolet source.

2.7. Kinetic analyses of the synthesome-mediated in vitro DNA replication in the absence or presence of Ara-CTP, CPT or DOX

DNA replication reactions mediated by the synthesome were performed in the absence or presence of ara-CTP, CPT, or DOX at concentrations required to inhibit the synthesome-mediated in vitro DNA replication reactions by 50%. Five hundred microliter reaction mixtures were incubated at 25 °C for 2 h. Aliquots (50 μl) were removed at 0, 1, 3, 5, 10, 15, 30, 60, 90, and 120 min intervals. DNA replication products were isolated by phenol–chloroform extraction and digested with the restriction endonuclease *Dpn* I (2 U, 1 h, 37 °C). The *Dpn* I resistant products were purified with Chroma Spin Columns (Clontech Laboratories, Inc.) according to the manufacturer's protocol. They were then treated with the combination of the restriction endonucleases indicated and the digestion products resolved from one another by electrophoresis through a 5% polyacrylamide gel containing TBE buffer. Gels were dried and the positions of the reaction products were visualized by autoradiography using Kodak XAR-5 films at −80 °C.

3. Results

3.1. Inhibition of synthesome-mediated in vitro DNA replication by Ara-CTP, CPT and DOX

We have previously shown that the IC_{50} value of ara-CTP required to inhibit the synthesome-driven in vitro DNA replication reaction was 65 μM [3]. In order to examine the effect of CPT and DOX on synthesome-mediated DNA synthesis, the in vitro DNA replication reactions were performed in the absence or presence of increasing concentrations of these drugs. Our results indicated that CPT and DOX at 0.25 and 0.75 μM, respectively, reduced in vitro DNA synthesis mediated by the synthesome by 50% (Table 1). In these experiments VCR, an inhibitor of microtubule assembly [19], did not show any inhibitory effect and was used as a negative control (Table 1).

Table 1
IC_{50} values of CPT and DOX required to inhibit synthesome-mediated in vitro DNA replication[a]

Compound	IC_{50} (μM)
CPT	0.25
DOX	0.75
VCR[b]	–

[a] Synthesome-mediated in vitro DNA replication was carried out as described previously [3,7] in the absence or presence of increasing concentrations of CPT, DOX, or VCR. DMSO was used as solvent for the stock solution of CPT. Reactions performed in the presence of DMSO were used as a control for the CPT experiments. Drug–dose response curves for CPT, DOX, and VCR were prepared by plotting the number of nanomoles of ^{32}P-dGTP incorporated into SV40 origin-containing plasmid (pSVO$^+$) during synthesome-driven DNA replication reactions at each drug concentration chosen. The IC_{50} value of each drug was determined from these drug dose-response curves. Data represent the mean of 3–5 independent experiments.

[b] VCR did not show any inhibitory effect on synthesome-mediated in vitro DNA replication at concentrations ranging from 0.01 to 100 μM and was used as a negative control in these experiments.

3.2. Neutral and alkaline gel analyses of the synthesome-mediated in vitro DNA replication products formed in the absence or presence of increasing concentrations of CPT or DOX

Ara-C and CPT have been shown to inhibit in vitro synthesome-driven DNA replication at concentrations that affect intact cell DNA synthesis [1,3]. In addition, our previous results with ara-CTP [3] have demonstrated that this drug inhibited DNA replication intermediates and Okazaki fragment formation in DNA replication reactions mediated by the synthesome. In this study, in order to evaluate how CPT and DOX affect synthesome-mediated DNA synthesis, the DNA replication products derived from the reactions performed in the absence or presence of these agents were analyzed by neutral and alkaline agarose gel electrophoresis. Under these conditions the replication intermediates were resolved on the basis of their molecular size and compactness. Our results demonstrate that the DNA synthesome is competent to support the formation of full-length form I (superhelical), form II (relaxed nicked circular), and form III (double-strand linear) DNA (Figs. 1A and 2A; Lane 1). In addition, concatemeric long DNA replication intermediates appeared as a smear extending from the top of the gel up to the position of form II DNA (Figs. 1A and 2A; Lane 1). In alkaline gels (i.e., under denaturing conditions), full-length daughter

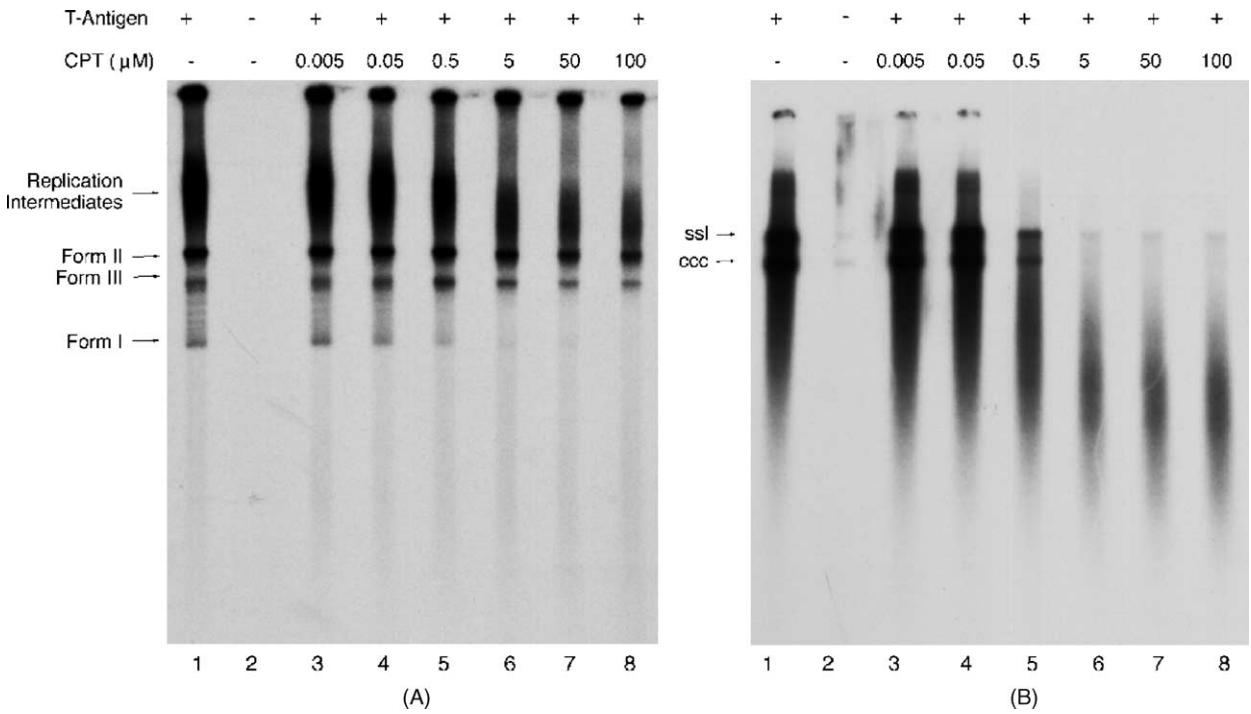

Fig. 1. (A) Neutral and (B) alkaline gel analyses of the synthesome-mediated in vitro SV40 DNA replication products formed in the absence or presence of increasing concentrations of CPT. The DNA replication products formed in the in vitro DNA replication reactions were isolated by phenol–chloroform extraction followed by precipitation with 2-propanol in the presence of 2 M ammonium acetate. The isolated DNA was suspended in 10 mM Tris/1 mM EDTA and electrophoresed through 1% agarose gels containing either (A) TBE or (B) alkaline buffers. Gels were dried and exposed to Kodak XAR-5 films at −80 °C for autoradiographic analysis of the resolved DNA replication products. Control experiments were carried out in the absence of CPT and in the presence of DMSO (4% final concentration). ssl, single-stranded linear DNA; ccc, covalently closed circular DNA.

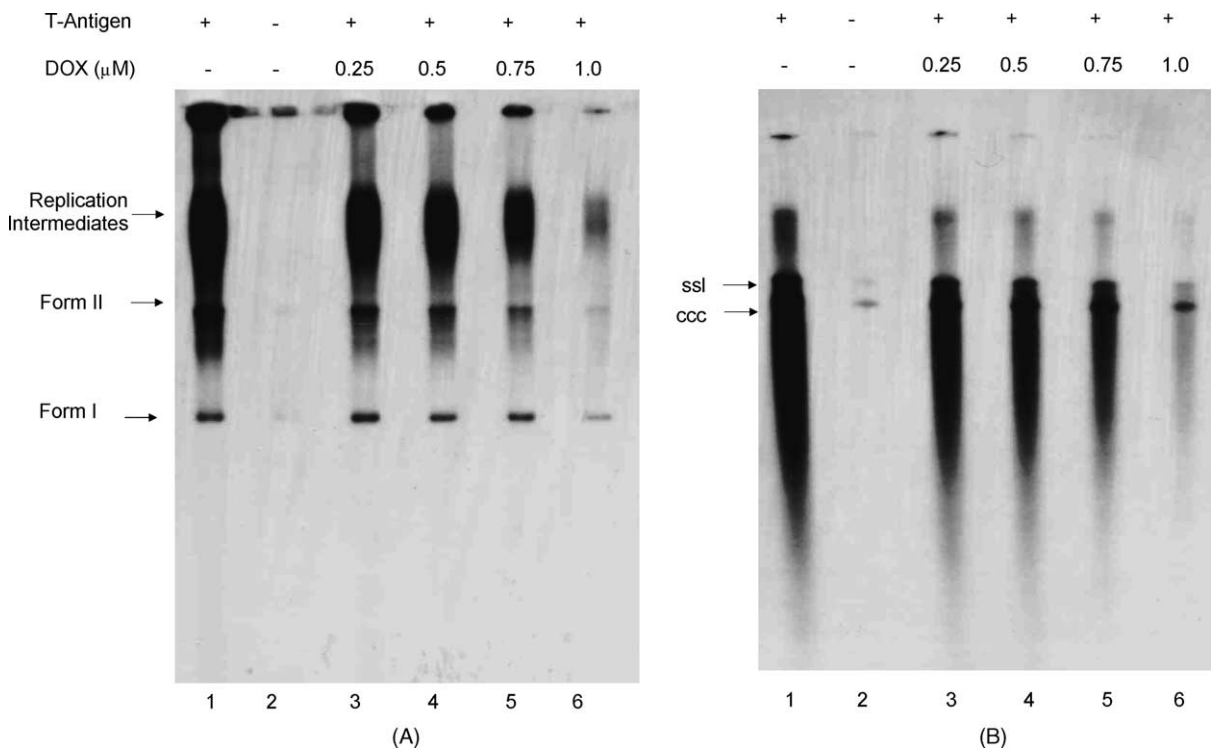

Fig. 2. (A) Neutral and (B) alkaline gel analyses of the synthesome-mediated in vitro SV40 DNA replication products formed in the absence or presence of increasing concentrations of DOX. The DNA replication products formed in the in vitro DNA replication reactions were isolated and electrophoresed through 1% agarose gels under either neutral or alkaline conditions. Gels were dried and exposed to Kodak XAR-5 films at −80 °C for autoradiographic analysis of the resolved DNA replication products. ssl, single-stranded linear DNA; ccc, covalently closed circular DNA.

DNA molecules are represented by the single stranded linear (ssl) and covalently closed circular (ccc) species (Figs. 1B and 2B; Lane 1).

In the presence of increasing concentrations of CPT, the synthesome-mediated in vitro DNA replication reaction was dramatically inhibited with the resulting suppression of full-length form I daughter DNA synthesis. The replication products migrated to the positions of the linear (form III) and the relaxed nicked circular (form II) DNA (Fig. 1A, Lanes 5–8). Alkaline gel analyses showed that CPT (at concentrations ranging between 0.5 and 100 μM) inhibited the synthesis of the long replication intermediates with subsequent accumulation of short DNA fragments (Fig. 1B, Lanes 5–8). DOX, on the other hand, showed only slight inhibitory effect on the formation of the long replication intermediates by the DNA synthesome, relative to control reactions lacking DOX, at concentrations ranging from 0.25 to 0.75 μM (Fig. 2A and B, Lanes 3–5). Strong inhibition of these catenated dimers and full-length DNA replication products appeared only when the reaction was carried out in the presence of 1.0 μM of the drug (Fig. 2A and B, Lane 6).

3.3. The development of a synthesome-based in vitro kinetic assay to study the effects of S-phase specific anticancer agents on the different stages of DNA replication

To perform a detailed analysis of the effects of ara-CTP, CPT, and DOX on synthesome-driven in vitro DNA replication, a novel kinetic assay was developed. Studies using the kinetic assay were performed to determine the precise step(s) in the DNA replication process (i.e., initiation, elongation, and termination) affected by each drug. The assay is based on the fact that in vitro SV40 DNA replication in cell extracts initiates from the viral origin of replication and proceeds bidirectionally from the origin in order to elongate the daughter DNA molecule until synthesis terminates halfway around the parent plasmid molecule.

The rationale for the kinetic assay was that the progress of DNA synthesis by the synthesome could be mapped as replication proceeded around a plasmid DNA containing an inserted viral origin sequence. In a synthesome-driven DNA replication reaction, the first DNA synthesis products arise from the viral replication origin and sequences adjacent to either side of these sequences. Later in the reaction, replication products would also bear DNA sequences distal to the viral origin. At still later times in the reaction, the replication products should contain DNA sequences that correspond approximately to opposite halves of the parental DNA molecule since the bidirectional replication process terminates when the two growing replication forks collide with one another approximately half-way around the circular DNA template molecule.

Dependence of the synthesome-mediated in vitro DNA replication reaction on the presence of the SV40 origin of replication was tested by performing the reaction using either pUC.HSO (ori^+), which contains the SV40 replication origin sequence or pUC.8–4 (ori^-) which is identical to pUC.HSO except for a 4 bp deletion within the SV40 origin [20]. Fig. 3 clearly demonstrates that the SV40 origin of replication and the SV40 large T antigen are essential for the DNA synthesome to form newly replicated form I DNA, form II DNA, and the higher order topological intermediates. Our results showed that DNA replication mediated by the synthesome starts from the SV40 origin of replication because no replication was detected with pUC.8–4 (ori^-) even in the presence of SV40 large T antigen (Fig. 3, compare Lanes 1, 3 and 5, 7).

In an attempt to map the bidirectional movement of synthesome-driven DNA replication, we made use of the plasmid $pSVO^+$ [21]. This plasmid is equivalent to pUC.HSO and contains a functional SV40 origin DNA sequence. Five different restriction endonucleases that make only a single cut in the $pSVO^+$ plasmid were used to digest the plasmid DNA (Fig. 4A). The restriction endonucleases used were *Psh*A I, *Ava* I, *Alw*N I, *Bsa* I, and *Sca* I. These restriction enzymes produced five DNA fragments ranging in size from 411 to 900 bp (Fig. 4B). The 200 bp DNA fragment representing the SV40 origin of replication was contained within the 900 bp (*Sca* I/*Psh*A I) fragment.

The first DNA products detected at early time points in the synthesome-driven DNA replication reaction are expected to be within the 900 bp (*Sca* I/*Psh*A I) fragment which contains the origin of replication. As the replication reaction proceeds with time, the newly replicated DNA products isolated from the reactions will correspond to nucleotide sequences that are further and further from the replication origin.

DNA replication mediated by the DNA synthesome showed a typical lag time of approximately 3–5 min before the detection of the first DNA products (900 bp) (Fig. 5A, Lanes 3 and 4). Synthesis of a complete daughter DNA molecule that can be detected on a polyacrylamide gel occurs within 1.5–2 h after initiation of the reaction (Fig. 5A, Lanes 9 and 10). The results also clearly demonstrated that synthesome-mediated in vitro DNA replication occurs bidirectionally; initiating from the origin and terminating halfway around the plasmid template molecule. The detection of the 900 bp (*Sca* I/*Psh*A I) fragment (that contains the origin of replication) was followed by detection of the adjacent DNA sequences (the 411 and 713 bp fragments). The 713 bp (*Psh*A I/*Ava* I) DNA fragment appeared after 10–15 min (Fig. 5A, Lanes 5 and 6) and was followed by the 411 bp (*Sca* I/*Bsa* I) piece 30 min after replication initiation (Fig. 5A, Lane 7). The detection of these fragments which were adjacent to the origin-containing fragment indicated that replication initiated at the origin and proceeded bidirectionally from that origin.

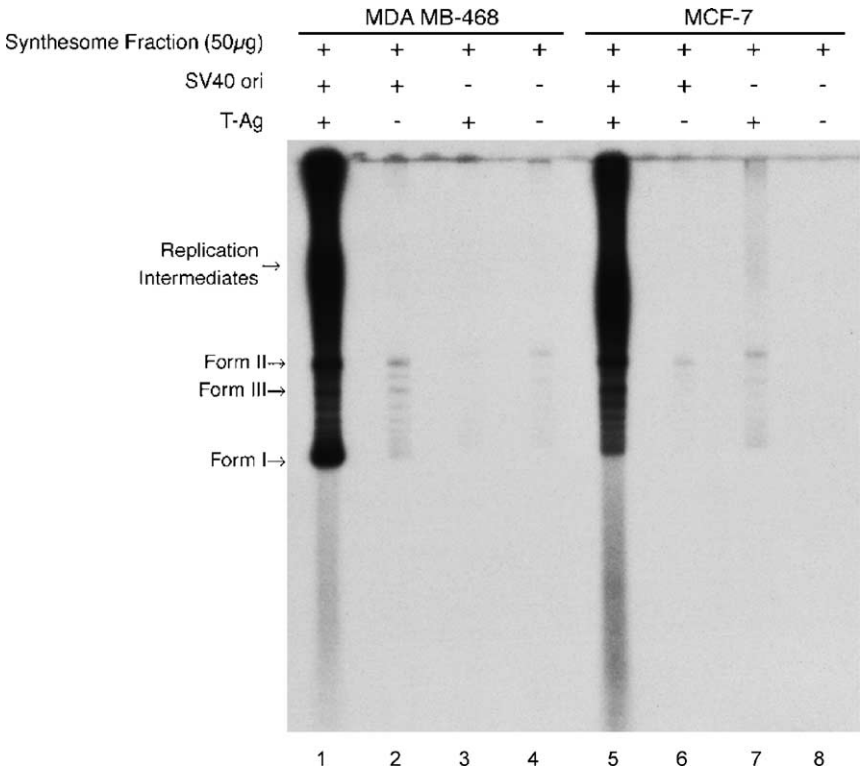

Fig. 3. Synthesome-mediated in vitro DNA replication depends on the presence of the SV40 origin of replication. In vitro DNA replication reactions were performed as described in the text using either pUC.HSO (ori$^+$) or pUC.8-4 (ori$^-$) plasmids. Lanes 1, 2, 5, and 6, reactions performed using pUC.HSO (ori$^+$) plasmid; Lanes 3, 4, 7, and 8, reactions performed using pUC.8–4 (ori$^-$) plasmid. MCF-7 and MDA MB-468 above the figure indicate the breast cancer cell line from which the DNA synthesome was isolated.

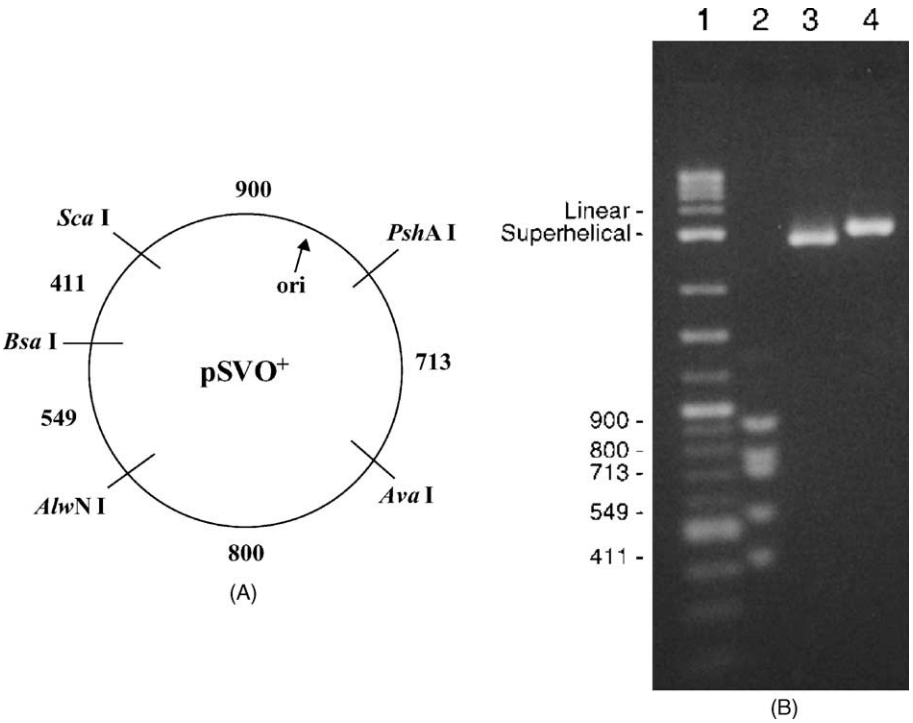

Fig. 4. (A) A map of the pSVO$^+$ plasmid restriction sites with the replication origin (ori) indicated within the 900 bp fragment. The cutting sites for five restriction endonucleases are shown. (B) Agarose gel electrophoresis (1.5% agarose in TBE) of the DNA digestion products after staining with ethidium bromide (0.5 μg/ml) and illumination with UV light. Lane 1, marker DNA containing a mixture of 100 bp and 1 kb DNA ladders; Lane 2, the five DNA fragments produced after digestion of the plasmid with the restriction endonucleases; Lane 3, pSVO$^+$ plasmid (supercoiled) without digestion; Lane 4, pSVO$^+$ after its linearization by *Ava* I enzyme. The numbers beside the panel indicate the sizes of the DNA fragments in bp.

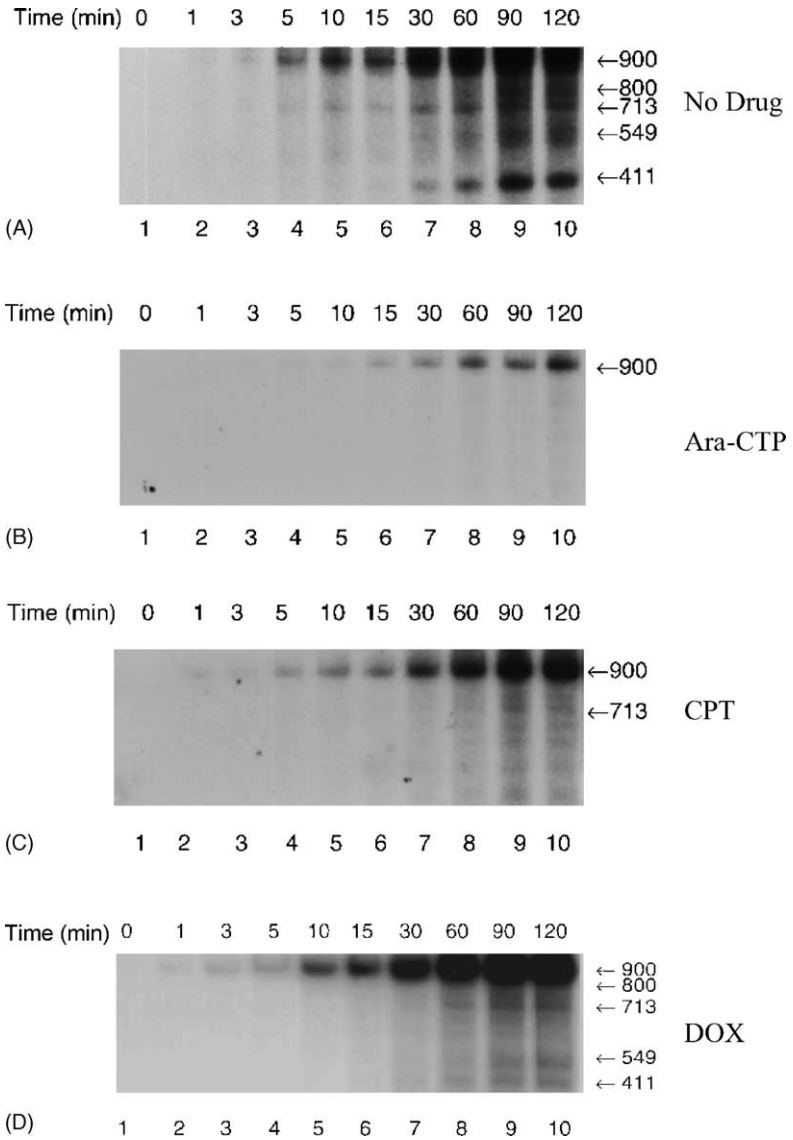

Fig. 5. Kinetic analyses of the synthesome-mediated in vitro DNA replication in the: (A) absence or (B) presence of ara-CTP, (C) CPT, or (D) DOX. DNA replication reactions were performed as described in the text in the (A) absence or (B) presence of the IC_{50} values of ara-CTP, (C) CPT, or (D) DOX. Five hundred microliter reaction mixtures were incubated at 25 °C for 2 h. Aliquots (50 μl) were removed at 0, 1, 3, 5, 10, 15, 30, 60, 90, and 120 min intervals. DNA replication products were isolated by phenol–chloroform extraction, treated with the combination of the restriction endonucleases, and electrophoresed in a 5% polyacrylamide gel containing TBE buffer. The gels were dried and exposed to Kodak XAR-5 films at −80 °C followed by autoradiography.

The 549 bp (*Bsa* I/*Alw*N I) DNA fragment then appeared after 60–90 min (Fig. 5A, Lanes 8 and 9), and eventually the 800 bp (*Ava* I/*Alw*N I) fragment together with the other four DNA fragments were detected between 90 and 120 min after starting the DNA replication reaction. The appearance of all five fragments of the plasmid template indicated that the synthesis of a full-length DNA molecule had occurred (Fig. 5A, Lanes 9 and 10).

3.4. Ara-CTP, CPT, and DOX affect different stages of synthesome-mediated in vitro DNA replication

Studies were then performed to determine the phases of synthesome-mediated DNA replication that were specifically inhibited by ara-CTP, CPT, and DOX. This was done by performing the synthesome-based DNA kinetic assay in the presence of these agents for various lengths of time. The assays were performed with drug concentrations inhibiting at least 50% of the DNA synthetic activity of the complex. An aliquot of the reaction mixture was removed at various time points and the DNA replication products were completely digested (using the combination of the restriction endonucleases listed in Fig. 4A) and resolved through a 5% polyacrylamide gel.

The time required to detect the 900 bp DNA fragment and to detect all of the five DNA fragments (indicating the synthesis of a full-length daughter DNA product) was determined. By comparing the length of time required to detect a specific DNA fragment produced during reactions performed in the absence or presence of the drugs, it

was possible to evaluate the specific effect these agents have on the rate of replication as well as define the precise stage(s) at which DNA synthesis was inhibited by each drug.

We expect ara-CTP to inhibit the initiation and elongation of DNA synthesis because ara-CTP inhibits the activity of both the leading and lagging strand DNA polymerases (DNA polymerases δ and α, respectively), with the inhibition of the lagging strand DNA polymerase activity being several fold greater than that of the leading strand DNA polymerase. As CPT is known to be a topoisomerase I inhibitor, which functions during the unwinding of the DNA template duplex, we expect CPT to slow the elongation reaction. DOX is known to inhibit the activity of topoisomerase II and as such is expected to have little inhibitory effect on the initiation and elongation phases of the DNA replication process. Unlike CPT, however, DOX is expected to inhibit the decatenation of newly replicated DNA, which should cause large replication intermediates composed of DNA concatemers to accumulate.

We have previously shown that 65 μM ara-CTP inhibited 50% of the synthesome-mediated in vitro DNA replication and Okazaki fragment synthesis [3]. When the kinetic assay was performed in the presence of this concentration of ara-CTP, there was a delay in the time required for detecting the 900 bp (*Sca* I/*Psh*A I) DNA fragment that contains the SV40 origin of replication. We could not detect this DNA fragment until 15–30 min after the initiation of DNA replication mediated by the synthesome (compare Fig. 5B, Lanes 6, 7 and Fig. 5A, Lanes 3 and 4). However, when the reaction was performed in the presence of either 0.25 μM CPT or 0.75 μM DOX, the 900 bp fragment, containing the SV40 origin, was detected within 5 min of initiating DNA replication (Fig. 5C and D, Lane 4). This suggests that these two agents did not affect the initiation stage of synthesome-driven in vitro DNA replication. All of the five DNA fragments could not be detected at the end of the 2 h period in the presence of ara-CTP or CPT indicating the inhibition of full-length daughter DNA synthesis (Fig. 5B and C; Lane 10). However, in the presence of DOX, the five DNA fragments were detected following a 90–120 min incubation (Fig. 5D, Lanes 9 and 10).

4. Discussion

Ara-C, CPT, and DOX are three widely used anticancer agents that directly affect different stages of DNA replication process. Incorporation of ara-C into replicating DNA and the subsequent inhibition of the initiation [22,23] and/or elongation [24,25] phases of the DNA synthetic process represent one of the mechanisms that might explain the antiproliferative effect of this drug [26,27]. Inhibition of DNA synthesis and the lethal effect of CPT, however, result from stabilization of the DNA–topoisomerase I cleavage complex [28–31]. The collision between the advancing replication fork and CPT-trapped topoisomerase I cleavage complex results in irreversible arrest of the replication fork movement and the generation of DNA double strand breaks which are lethal to the cell. DOX, an anthracycline antibiotic, is active against several kinds of malignancies including leukemia, breast, small cell lung, and ovarian cancers. It exerts its lethal effect and poisons topoisomerase II enzyme by increasing the concentration of its covalent DNA cleavage complexes, thereby converting topoisomerase II into a physiological toxin [32–34]. This action creates double-stranded DNA breaks in the cellular genome and triggers several mutagenic and lethal events [32,35].

In this study, we tested the DNA synthesome for its ability to correctly predict the anticipated effects of ara-CTP, CPT and DOX on the DNA replication process. Our studies validated the usefulness of the DNA synthesome as an in vitro model system for analyzing the molecular mechanism of S-phase specific anticancer agents. We initiated experiments to examine the effects of ara-CTP, CPT, and DOX on the DNA replication process mediated by the DNA synthesome. Daughter DNA molecules produced in the in vitro synthesome-driven DNA replication reactions in the absence or presence of increasing concentrations of these agents were analyzed by neutral and alkaline gel electrophoresis. Our results showed that the DNA synthesome is fully competent to support SV40 origin-specific and large T antigen-dependent DNA replication in vitro. The DNA replication products detected included form I, form II, as well as concatemeric and higher order topological intermediates. These forms are identical to those obtained either from SV40 virus infected cells [36–38] or from in vitro DNA replication of SV40 origin containing plasmids using crude cell extracts [20,39,40].

We have previously shown that ara-CTP inhibits formation of cancer cell DNA replication intermediates and Okazaki fragment synthesis in in vitro DNA replication reactions mediated by the synthesome [3]. In the presence of increasing concentrations of CPT, the formation of daughter DNA molecules was dramatically suppressed. Inhibition of synthesome-mediated in vitro DNA replication was detected at CPT concentrations higher than 0.05 μM. Using HeLa cell cytosolic extracts, Hsiang et al. [41] and Tsao et al. [42] showed that CPT inhibits in vitro DNA replication reactions after the addition of calf thymus topoisomerase I to the extract system. Our results are consistent with those of Ishimi et al. [43] who found that in vitro DNA replication using HeLa cell extract was inhibited by CPT with the generation of short nascent DNA replication products.

In contrast to CPT, neutral and alkaline gel analyses of synthesome-mediated DNA replication products in the presence of increasing concentrations of DOX indicated that this drug did not inhibit the formation of long DNA replication intermediates at concentrations ranging from 0.25 to 0.75 μM. Inhibition of the synthesis of catenated

dimers and full-length DNA molecules appeared only at higher concentrations of the drug. The topoisomerase II enzyme plays an important role as a swivelase in the late stage of SV40 DNA replication [43,44] and in the separation of the newly replicated DNA molecules [37,45,46]. Inhibition of the topoisomerase II enzyme, therefore, results in slowing of late SV40 DNA replication and interference with the segregation of daughter DNA molecules. Richter et al. [47] and Richter and Strausfeld [48] examined the effect of teniposide (VM-26) on SV40 DNA replication both in vivo and in vitro. They demonstrated that inhibition of topoisomerase II by this drug leads to accumulation of long replication intermediates. Our results are in accordance with those of Snapka [45] and Snapka et al. [49] who showed that treatment of SV40-infected CV-1 cells with doxorubicin resulted in accumulation of highly catenated dimers.

In this study, we conducted detailed kinetic analyses of in vitro synthesome-mediated DNA replication reactions in the absence or presence of ara-CTP, CPT, or DOX. We developed a synthesome-based kinetic technique in order to examine how these anticancer agents affect different stages of DNA replication mediated by the complex. Our results clearly demonstrate that DNA synthesis mediated by the synthesome is completely dependent on the presence of the SV40 replication origin since we could not detect any daughter DNA molecules when using an ori$^-$ template in our assay. Our results also showed that synthesome-mediated in vitro DNA replication initiates from the origin and proceeds bidirectionally from the initiation site. The DNA synthesome initiates DNA replication after a lag time of approximately 5 min and synthesis of complete daughter molecules occurs within 90–120 min after initiating the reaction.

By comparing the length of time required to detect the DNA fragment containing the SV40 origin of replication to that required to detect other DNA fragments formed in the reaction carried out in the absence or presence of ara-CTP, CPT, or DOX, it became possible to evaluate the specific effect these agents had on the rate of replication. It also became possible to define the precise DNA synthesis stage(s) inhibited by each drug. In the presence of ara-CTP, initiation of DNA replication mediated by the synthesome was delayed since the DNA origin fragment was detected after 15–30 min following the initiation of the reaction. In addition, this was the only DNA fragment detected during the 2 h assay period. This result suggests that ara-CTP suppresses synthesome-mediated in vitro DNA replication through its inhibition of the initiation and early stages of DNA chain elongation. The stronger inhibitory effect of this compound on the synthesome-associated DNA polymerase α activity supports this contention.

Ara-C has been reported to inhibit the elongation phase of DNA replication [25,50]. Our previous study [3] utilizing the DNA synthesome showed that ara-CTP inhibits both the initiation of DNA replication and Okazaki fragment synthesis. This effect was accompanied by a preferential inhibition of synthesome-associated DNA polymerase α activity [3,5]. Our findings are supported by the studies of Fridland [22,23] and Bell and Fridland [51] that suggested that incorporation of ara-CMP into DNA was correlated with inhibition of the initiation of new DNA synthesis.

Our results also showed that CPT did not affect the initiation phase of DNA replication mediated by the synthesome as might be expected. Topoisomerase I has been shown to be involved in the initiation of SV40 DNA replication [52]. However, it has also been shown that topoisomerase II activity can substitute for the topoisomerase I enzyme in a reconstituted SV40 replication system [53]. Our results suggest that since topoisomerase II co-purifies with the DNA synthesome [8], it may compensate for CPT inhibition of topoisomerase I during the initiation phase of synthesome-mediated in vitro DNA replication. The results presented here, which showed inhibition of the elongation stage of synthesome-mediated DNA replication in vitro by CPT, support this suggestion. We have previously studied the effect of advancing replication fork collision with the cleavage complex on both the leading and lagging strands of DNA synthesis [54]. The results of this study indicated that the formation of a topoisomerase I cleavage complex in response to CPT treatment results in replication runoff on the leading strand with subsequent formation of DNA double-strand breaks. Replication-mediated double-strand breaks, however, were not detected on the lagging strand. Based upon the data presented here and that of Strumberg et al. [54], we believe that the replication fork might be arrested upstream of the CPT-trapped topoisomerase I cleavage complex.

Using the synthesome-based kinetic assay we have also shown that DOX neither affected the initiation nor the elongation stages of the DNA replication reaction mediated by the synthesome. This conclusion is based on the fact that we detected the 900 bp DNA fragment, containing the SV40 origin of replication, within 5 min of initiating the reaction and that full-length daughter DNA molecules were formed during the incubation period. This result is in accordance with the fact that topoisomerase II enzyme is essential for the segregation of fully replicated circular DNA molecules (i.e., the termination stage of the SV40 DNA replication cycle). The effect of DOX on the kinetics of DNA replication mediated by the synthesome in addition to its effect on synthesome-mediated in vitro DNA replication and the accumulation of long replication intermediates suggest that this drug acts mainly by inhibition of the termination phase of the DNA synthesis process.

Our synthesome-based SV40 model system has several advantages that make it an ideal in vitro model to study S-phase anticancer drug action. The synthesome can mediate the initiation, elongation, and termination phases of the eukaryotic DNA replication process in vitro. This model permits the accurate assessment of whether an anticancer drug has the ability to directly interact with the DNA

synthetic process while the process is uncoupled from any other cellular events that could secondarily affect DNA replication. In addition, the use of the synthesome permits the investigator to examine the anticancer drug's ability to affect the entire DNA replication apparatus, and not just isolated individual protein components that have a role in the DNA synthetic process.

Collectively, the results of our studies suggest that the in vitro SV40 DNA replication model system utilizing the human cell DNA synthesome may serve as an accurate representation of the cellular DNA replication machinery making it valuable for investigating the mechanism by which anticancer agents directly affect the process of DNA replication.

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